Pancreas organogenesis: the interplay between surrounding microenvironment(s) and epithelium-intrinsic factors

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Abstract

During embryonic development, pancreatic epithelial cells engage in concomitant morphogenetic and fate specification events that will give rise to the final organ architecture and functions. Cues from the surrounding microenvironment are known to influence the behaviour of epithelial progenitors and orchestrate these concomitant events throughout pancreas development. Nevertheless, the composition of the pancreatic microenvironment remains elusive; also, the interplay between components of the surrounding microenvironment and the epithelium is poorly characterized.

We present here a comprehensive overview of the pancreatic microenvironment and what is known regarding distinct cell types, signaling molecules, ECM, that constitute it. We focus on the molecular circuits governing cell-cell interactions, which are at play in the developing pancreas, controlling pancreatic progenitor proliferation, morphogenesis, and differentiation. Finally, open questions and implication of future research in this field are discussed in the context of pancreatic diseases, such as diabetes and cancer, as well as therapeutic approaches for these diseases.

The adult pancreas is a composite organ responsible for two vital functions in our body: production of digestive enzymes and glucose metabolism (Slack, 1995). The exocrine portion of the pancreas is composed of acinar and ductal cells, which produce and collect, respectively, the enzymes that facilitate nutrient absorption in the gut. The acinar cells secrete important enzymes for the catalysis of proteins, lipids and carbohydrates. The bicarbonate- and mucin-secreting ductal cells form a complex ductal network allowing the release of the pancreatic secretions into the duodenum. The endocrine compartment is organized into the islets of Langerhans and is responsible for the production and secretion of glucose modulating hormones in the systemic circulation. The most abundant cell type in human and mouse islets are the insulin-producing β-cells (≥80%); the remaining fraction includes glucagon-producing α-cells, somatostatin-producing δ-cells and pancreatic polypeptide-producing PP cells (Pan and Wright, 2011; Slack, 1995). Islet-secreted hormones play a crucial role in the regulation of glucose homeostasis (Romer and Sussel, 2015).

The vital functions controlled by the pancreas are reflected in the morbidity and mortality associated with pancreatic diseases, such as diabetes and pancreatic cancer (Ellis et al., 2017; Feig et al., 2012; Pociot and Lernmark, 2016; Zheng et al., 2018). Diabetes is generally characterized by hyperglycemia due to loss or dysfunction of the endocrine β-cells (Pociot and Lernmark, 2016). The challenge to curing diabetes relies on the replacing the lost or damaged insulin-producing β-cells in diabetic patients (Sneddon et al., 2018). On the other hand, the exocrine compartment is the target of pancreatitis and pancreatic ductal adenocarcinoma (PDAC), which is a devastating malignancy with an extremely poor prognosis and unmet medical need (Feig et al., 2012; Sinha and Leach, 2016). Work from the past two decades clearly showed how much the understanding of the mechanisms underlying pancreas formation has helped elucidating the pathogenesis of pancreatic

diseases and developing therapeutic strategies for these diseases. Hence, remarkable advances in generating a sustainable source of functional human stem cell-derived insulin-producing cells for cell transplantation have been made thanks to the identification of developmental factors, which are released from the pancreatic microenvironment and regulate expansion and differentiation of pancreatic cells (Sneddon et al., 2018). Moreover, aberrant activation of embryonic signaling pathways is frequent in PDAC, making developmental regulators therapeutically attractive (Biankin et al., 2012; Roy et al., 2016).

In this review, we focus on the pancreatic microenvironment and its different components with a particular emphasis on their interplay with the epithelium during pancreas organogenesis. We draw mainly from findings in the mouse model, but that are conserved in other model organisms, including in humans. We discuss the implications of these findings in the context of pancreatic diseases and therapeutic approaches.

Overview of embryonic pancreas development

Many excellent and comprehensive reviews have been written on the transcriptional control underlying pancreas development (Arda et al., 2013; Jørgensen et al., 2006; Larsen and Grapin-Botton, 2017; Pan and Wright, 2011; Shih et al., 2013; Slack, 1995). Here, we provide only a brief overview of the key morphogenetic events and transcription factors (TF) important for establishing pancreatic cell identity.

The pancreas, together with the liver, biliary system, lung, thymus, thyroid and epithelial cells lining the respiratory and digestive systems, originates from the endoderm germ layer (Slack, 1995; Spagnoli, 2007; Zorn and Wells, 2007). Pancreatic fate specification occurs in the mouse embryo at approximately embryonic day (E)8.5-8.75, when the expression of the homeodomain TF *Pancreatic and duodenal homeobox 1* (*Pdx1*) starts in two distinct

domains of the anterior region of the gut endoderm (*a.k.a.* foregut): the ventral and dorsal *anlagen* (Slack, 1995; Spagnoli, 2007; Zorn and Wells, 2007).

Commitment of endodermal cells to a pancreatic fate is a multistep process, involving a continuous crosstalk between the endoderm and surrounding tissues that eventually leads to the activation of a pancreatic gene expression program (Gittes, 2009; Spagnoli, 2007). At E9.5-10.5 in the mouse, mesenchymal cells condense around the two pancreatic buds, which consist of a pseudostratified epithelium positive for Pdx1 (Gittes, 2009; Landsman et al., 2011). Shortly after, at E11.5 pancreatic morphogenesis starts, allowing the pancreas to acquire the characteristic tree-like epithelial structure composed of a network of well-formed branches and connected ducts, necessary to the organ to fulfill its exocrine functions (Larsen and Grapin-Botton, 2017; Shih et al., 2013). Importantly, branching morphogenesis has been shown to be tightly coupled, both in a mechanistic and temporal manner, with growth and differentiation of the pancreatic cells (Larsen and Grapin-Botton, 2017; Villasenor et al., 2012). The first step of this complex process is the acquisition of an apico-basal polarity from the pancreatic epithelial cell, which is dependent on the Rho GTPase Cdc42 (Kesavan et al., 2009). Subsequently, some of these cells undergo apical constriction to form rosette structures with microlumens at the center (Hick et al., 2009; Villasenor et al., 2012). Microlumen formation and subsequent epithelial remodeling further depends on Eph/ephrin signalling (Villasenor et al., 2012). Moreover, actin cytoskeleton remodeling underlies apical constriction in pancreatic progenitors; this is regulated by Rho and the RhoGAP Stard13, whose disruption leads to reduced epithelial branching and associated pancreatic growth defects in the mouse (Petzold et al., 2013). Around E12.5, the fusion of the microlumina allows the establishment of primary branches, which subsequently undergo a combination of lateral branching and tip-splitting to generate a complex tubular plexus (Hick et al., 2009; Pan and Wright, 2011; Puri and Hebrok, 2007; Villasenor et al., 2012). This coincides with a gradual spatial segregation of the multipotent

pancreatic progenitor cells (MPC) and establishment of distinct pancreatic lineages. MPCs initially co-express a set of pancreatic TFs, including *Pdx1, Pancreatic-specific Transcription Factor 1a (Ptf1a), SRY-box 9* (*Sox9*), and *Hepatocyte nuclear factor 1β* (*Hnf1β*) (Ahlgren et al., 1997; Arda et al., 2013; Haumaitre et al., 2005; Kawaguchi et al., 2002; Offield et al., 1996; Seymour et al., 2007). After E12.5, pancreatic epithelial cells organize themselves in two different domains, called "tip" and "trunk" domains: the tip domain is marked by the expression of *Ptf1a*, *Gata4, c-myc* and *Cpa1,* and gives rise to acinar cells (Pan and Wright, 2011; Zhou et al., 2007), whereas the trunk region contains bipotent cells that are committed to become ductal and endocrine cells and express *Nkx6 homeobox 1 (Nkx6.1), Nkx6.2, Sox9, Hnf1β, Gata6* and *Hairy and enhancer of split (Hes1)* (Haumaitre et al., 2005; Henseleit et al., 2005; Jensen et al., 2000; Sander et al., 2000; Seymour et al., 2007; Solar et al., 2009). The mechanisms responsible for this early regionalization and underlying signaling pathways remain unknown. In other developing organ systems, focal sources of morphogens in the surrounding mesenchyme were shown to establish distinct gene expression domains (Hogan, 1999). If and how the pancreatic mesenchyme establishes "niche-like" environments responsible for distinct differentiation program and tissue-regionalization remain open questions.

Subsequently, following gut rotation, the ventral bud moves distally and fuses with the dorsal bud to form a single nascent pancreas (Jørgensen et al., 2006; Slack, 1995). This coincides with the major wave of pancreatic cell differentiation in a process known as "secondary transition" (Larsen and Grapin-Botton, 2017; Pan and Wright, 2011; Shih et al., 2013). Acinar cells at the tips of the branches begin expressing digestive enzymes, such as *Elastase* and *Amylase* (Pan and Wright, 2011). Within the trunk compartment, bipotent progenitor cells are directed into endocrine fate by induction of *Neurogenin 3 (Ngn3)*, whereas those that do not activate *Ngn3* will differentiate along the ductal compartment pathway (Gouzi et al., 2011; Magenheim et al., 2011b; Solar et al., 2009). Once *Ngn3*

transcription has initiated, it triggers exit from the cell cycle and delamination from the trunk into the surrounding mesenchyme where the endocrine progenitors will differentiate into the five different endocrine cell types (Gouzi et al., 2011; Larsen and Grapin-Botton, 2017; Miyatsuka et al., 2011). How the activation or repression of *Ngn3* in ductal cells is mechanistically achieved is still under active investigation (Bankaitis et al., 2015; Larsen and Grapin-Botton, 2017; Löf-Öhlin et al., 2017). The level of Notch signaling has been demonstrated to exert antagonistic effects on *Neurog3* transcription. The Notch target and effector *Hes1* has been shown to repress *Ngn3* transcription and accelerate Ngn3 protein degradation (Jensen et al., 2000; Larsen and Grapin-Botton, 2017; Qu et al., 2013); but, how different trunk cells acquire different Notch activation levels is still unknown. *Ngn3* activates a set of TFs, which, in turn, control further differentiation of endocrine progenitor cells into the five endocrine cell types, such as the LIM homeobox transcription factor *Islet1* (*Isl1*), *Nkx2.2, Insulinoma-associated 1 (Insm1), Rfx6* and *Neurogenic differentiation 1 (NeuroD1)* (Ahlgren et al., 1997; Mastracci et al., 2013; Osipovich et al., 2014; Romer and Sussel, 2015).

Of note, the majority of these TFs plays multiple roles in pancreatic development as well as in the adult organ, implying that the same TF can exert different functions depending on the cellular context. For instance, TFs that are required for β-cell differentiation, such as Pdx1 and Nkx6.1, must be actively maintained throughout the cell's lifetime; indeed, their loss triggers β-cell de-differentiation or switch of identity (Gao et al., 2014; Romer and Sussel, 2015; Taylor et al., 2013). Importantly, the same events as well as transcriptional silencing of these TFs have recently emerged to be associated with β-dysfunction and Type 2 diabetes (T2D) in humans (reviewed in (Romer and Sussel, 2015), stressing the relevance of further elucidating the islet-cell specific transcriptional networks.

The pancreatic microenvironment

During embryonic development, the pancreatic microenvironment is composed of multiple cell types, including mesenchymal cells, endothelial cells, neural crest-derived and immune cells, as well as structural molecules, which are part of the extracellular matrix (ECM) (Azizoglu and Cleaver, 2016; Gittes, 2009; Hisaoka et al., 1993; Landsman et al., 2011; Nekrep et al., 2008; Pierreux et al., 2010; Shih et al., 2016) (Fig. 1). Most of these components may exert distinct roles at subsequent steps of pancreatic development, influencing fate specification, growth, morphogenesis and/or differentiation. External inputs from the pancreatic microenvironment, including signaling effectors and cell interactions, and their impact(s) on pancreatic epithelial cells at different developmental stages are discussed in the following sections.

Early tissue interactions underlying pancreatic fate specification

Studies in different vertebrate species have shown that during gastrulation instructive signals from the adjacent germ layers, including Fibroblast Growth Factor (FGF), Retinoic Acid (RA), Bone Morphogenetic Protein (BMP), render the endoderm competent to receive subsequent pro-pancreatic signals signaling effectors (Spagnoli, 2007; Zorn and Wells, 2007). After gastrulation, once the foregut endoderm has completed its ventral closure, the prospective pancreatic endoderm is exposed to a series of tissue interactions, which are required for its fate specification (Pan and Wright, 2011; Shih et al., 2013; Spagnoli, 2007). Interestingly, because the organ arises from two separate endoderm domains, different tissue interactions are established during early development. Specifically, the dorsal bud is in contact with the notochord and dorsal aorta, while the ventral buds with lateral plate mesoderm, cardiac mesoderm and vitelline veins (Deutsch et al., 2001; Gittes, 2009; Hebrok et al., 1998; Kim et al., 1997; Kumar et al., 2003; Lammert et al., 2001; Yoshitomi and Zaret, 2004). Despite these contrasting local niches and some transcriptional

heterogeneity revealed by bulk and single-cell RNASeq (Li et al., 2018; Rodríguez-Seguel et al., 2013), both pancreatic buds possess the ability to differentiate into all pancreatic cell types, suggesting that different developmental cues can be used to promote pancreatic fate.

The notochord lies next to the dorsal midline endoderm and remains in contact with the dorsal pancreatic endoderm until E8 in the mouse embryo, when it is displaced by the fusion of the paired dorsal aortas (Lammert et al., 2001; Slack, 1995). Seminal studies in the chick showed that the notochord is required and sufficient for pancreatic fate induction (Hebrok et al., 1998; Kim et al., 1997). Removal of the notochord in explanted chick embryos prevents dorsal pancreas formation, due to an ectopic expression of *Sonic Hedgehog* (*Shh)* in the pancreatic region (Hebrok et al., 1998; Kim et al., 1997). Conversely, grafting an ectopic notochord adjacent to the ventral foregut in a chick embryo results into suppression of endodermal *Shh* together with induction of pancreas formation. Additionally, these studies discovered that Activin-βB and FGF2 are the morphogens released by the chick notochord and responsible for repression of *Shh* expression in the pre-pancreatic region, allowing the initiation of pancreatic gene expression (Hebrok et al., 1998; Kim et al., 1997). Consistently, mouse mutants for ActRIIA and ActRIIB activin receptors exhibit pancreatic hypoplasia and defects in foregut patterning (Kim et al., 2000). Finally, gain-of-function studies of the Shh pathway in the mouse further established its role as repressor of pancreatic fate. Specifically, inactivation of two Hedgehog inhibitors, the receptor *Patched 1* (*Ptch1*) and *Hedgehog-interacting protein* (*Hhip)*, leads to increased Hedgehog signaling and disruption of pancreas formation (Kawahira et al., 2003; Kawahira et al., 2005). Also, ectopic expression of *Shh* in the pancreatic endoderm induces partial conversion of the endoderm into duodenal fate and change in identity of the surrounding pancreatic mesenchyme (Apelqvist et al., 1997).

Subsequently, around E9 in the mouse embryo, the paired dorsal aortae start to fuse at the midline and become interposed between the notochord and the dorsal region of the foregut endoderm (Azizoglu and Cleaver, 2016; Lammert et al., 2001). The newly established contact with endothelial cells is important for proper dorsal pancreas formation (Lammert et al., 2001; Yoshitomi and Zaret, 2004). *In vitro* tissue recombination experiments showed that aortic endothelial cells induce *Pdx1* and *insulin* expression in the adjacent dorsal endoderm, together with formation of structures resembling pancreatic tissue (Lammert et al., 2001). However, *in vivo* studies in mouse embryos null for *Flk1* [encoding Vascular Endothelial Growth Factor receptor (VEGFR) 2], which lack all endothelial cells, indicated that aortic endothelial cells are not required for the induction of *Pdx1* in the dorsal foregut, but for the later *Ptf1a* expression and expansion of the dorsal bud (Jacquemin et al., 2006; Yoshitomi and Zaret, 2004). Moreover, stimuli produced by the aorta are essential for the survival of the dorsal mesenchyme, which in turn influences dorsal pancreas development (Jacquemin et al., 2006; Yoshitomi and Zaret, 2004).

Retinoic acid (RA) also exerts a conserved role in pancreatic fate induction of the dorsal endoderm but not of the ventral bud (Arregi et al., 2016; Chen et al., 2004; Kumar et al., 2003; Martín et al., 2005; Stafford and Prince, 2002) (see below in “The pancreatic mesenchyme” section).

The ventral region of the foregut is exposed to a different set of interactions, including with the lateral plate mesoderm and its derivatives, the cardiac mesoderm and septum transversum mesenchyme as well as to the vitelline veins (Azizoglu and Cleaver, 2016; Deutsch et al., 2001; Gualdi et al., 1996; Lammert et al., 2001). These unique interactions are primarily important for the lineage restriction between liver and pancreas cell fate within the ventral foregut endoderm (Deutsch et al., 2001; Gualdi et al., 1996; Wandzioch and Zaret, 2009). The ventral foregut endoderm contains a putative bipotent hepato-pancreatic progenitor population, which by default is fated to adopt a pancreatic fate

(Angelo et al., 2012; Deutsch et al., 2001; Rodríguez-Seguel et al., 2013; Tremblay and Zaret, 2005). Mouse foregut explants cultured *in vitro* activate pancreatic gene expression, while exposure to FGF- and BMP-mediated signalings from the cardiac mesoderm induces hepatic fate (Deutsch et al., 2001; Gualdi et al., 1996; Rossi et al., 2001). More specifically, BMP inhibition has been suggested to be necessary for inducing pancreatic cell fate, whereas both BMP and FGF signaling are required for hepatic cell fate acquisition within the ventral foregut in a conserved fashion across species (Chung et al., 2008; Deutsch et al., 2001; Rossi et al., 2001; Spagnoli and Brivanlou, 2008). Zaret and colleagues discovered that the TF *Hematopoietically expressed homeobox gene* (*Hex)* helps to define the position of the prospective ventral pancreatic endoderm with respect to the cardiac mesoderm in the mouse (Bort et al., 2004). In the absence of *Hex,* ventral pancreatic progenitors fail to proliferate beyond the cardiac mesoderm to escape its hepatic-inducing signals and be specified (Bort et al., 2004). The establishment of proper levels of Wnt/β-catenin signaling and its temporal sequential activation in the anterior endoderm are also known to be essential for foregut identity and organ formation (Li et al., 2008; Ober et al., 2006; Rodríguez-Seguel et al., 2013). Recent observations identified the non-canonical Wnt pathway as a developmental regulator of the liver *versus* pancreas fate decision, promoting pancreatic fate in the endoderm of *Xenopus* embryos and mouse ES cultures (Rodríguez-Seguel et al., 2013).

The Hh pathway needs to be repressed during ventral pancreas fate specification, like in the dorsal bud, however this must occur *via* a notochord-independent mechanism (Gittes, 2009; Hebrok et al., 2000). Recent observations from Sussel and colleagues showed that *Shh* expression is upregulated in the pancreatic endoderm upon *Gata4/6* gene ablation, which results in arrested pancreatic development and cell fate switch of ventral pancreatic cells into intestinal fate and dorsal pancreas into stomach (Xuan and Sussel, 2016). This suggests a repressive interaction between Shh and the Gata4/6 TFs, representing a

unifying mechanism for dorsal and ventral pancreas fate specification (Xuan and Sussel, 2016).

The pancreatic mesenchyme

Mesenchymal cells derived from the splanchnic mesoderm condense around the evaginating pancreatic epithelium at E10 in the mouse (Gittes, 2009; Landsman et al., 2011). Seminal studies in the 1960s based on *ex vivo* tissue recombination experiments demonstrated the absolute requirement for pancreatic mesenchyme in epithelial growth and morphogenesis (Golosow and Grobstein, 1962; Wessells and Cohen, 1967). Subsequent *ex vivo* organ culture studies also revealed a critical role for the mesenchyme in pancreatic cell differentiation (Attali et al., 2007). Specifically, “naked epithelium” cultured in the absence of pancreatic mesenchyme sustains islet growth, while the presence of the pancreatic mesenchyme is required for induction of acinar fate (Miralles et al., 1998b). Only recently, Landsman *et al*. provided the first *in vivo* genetic evidence for the requirement of pancreatic mesenchyme throughout pancreas organogenesis in the mouse (Landsman et al., 2011). Combined use of Cre-lox technology with diphtheria toxin (DT)-induced apoptosis enabled tissue-specific depletion of pancreatic mesenchymal cells at different developmental stages *in vivo* (Landsman et al., 2011). Mesenchymal cells ablation at the onset of pancreas development results into pancreas agenesis, whereas depletion of mesenchymal cells at later stages leads to pancreatic hypoplasia and impaired epithelial morphogenesis, due to reduced epithelial proliferation (Landsman et al., 2011).

Even though the relevance of the mesenchyme during pancreatic organogenesis is well established, its embryonic origin is still elusive. Recent DiI fate mapping in mouse embryos cultured *ex vivo* suggests a common origin of dorsal and ventral pancreatic mesenchyme, both arising from a portion of the coelomic mesothelium (Angelo and Tremblay, 2018).

Yet, a common origin of the pancreatic mesenchyme is at odds with the differences reported so far between the transcriptional programs of dorsal and ventral mesenchyme(s). For instance, the TF *Isl1* is expressed in the dorsal mesenchyme, but not in the ventral one (Ahlgren et al., 1997). Consistently, *Isl1* gene deletion in the mouse leads to failure of dorsal mesenchyme development, which in turn affects dorsal Pdx1+ endoderm outgrowth and differentiation (Ahlgren et al., 1997). Similarly, *N-cadherin* (encoded by *Cdh2*) is expressed at high levels in the dorsal pancreatic mesenchyme starting from E9.5 and, subsequently, in the endocrine progenitors at E12.5, once delamination has started (Esni et al., 2001). Genetic ablation of *Cdh2* results into dorsal pancreas agenesis due to impaired dorsal pancreatic mesenchyme survival (Esni et al., 2001). Despite the similarities in expression pattern and mutant phenotypes, Isl1 and N-cadherin seem to function independently, since *Isl1* expression is unchanged in N-cadherin-deficient mesenchyme (Esni et al., 2001). Future genetic lineage studies are required to further define the origin of the pancreatic mesenchyme and clarify if ventral and dorsal mesenchyme(s) share a common origin; the Dil-labeling results does not exclude the possibility of an additional mesenchymal source, as previously reported in other organs (Kfoury and Scadden, 2015; Lee et al., 2017; Zepp et al., 2017).

The dorsal pancreatic mesenchyme not only is crucial for the development of the dorsal pancreatic epithelium, but it also contributes to spleen formation (Asayesh et al., 2006; Hecksher-Sørensen et al., 2004; Koss et al., 2012). Notably, the TFs *Nkx3.2* and *Nkx2.5,* which initially mark dorsal pancreatic mesenchymal cells, become restricted to the spleen at E12.5, and mice mutants for these genes are asplenic (Asayesh et al., 2006; Koss et al., 2012). Specifically, in the absence of *Nkx3.2* the splenic mesenchyme fails to condense and does not segregate from the pancreas, resulting in metaplastic transformation of the pancreatic epithelium and mesenchyme into intestinal-like tissue, possibly due to ectopic *Shh*-espression (Asayesh et al., 2006; Hecksher-Sørensen et al.,

2004). Interestingly, rare human congenital disorders present concurrent splenic defects (asplenia or polyspenia) and dorsal pancreatic aplasia/hypoplasia is likely to be explained by the common origin of the splenic and pancreatic mesenchyme (Drut et al., 1993; Hadar et al., 1991; Herman and Siegel, 1991; Kim et al., 2009; Low et al., 2011; Sener and Alper, 1994; Sriplung, 1991; Wainwright and Nelson, 1993).

Mesenchymal signaling molecules

To date, several signaling molecules have been identified to be released by pancreatic mesenchymal cells and participate in the epithelial-mesenchymal crosstalk during pancreatic development. Often these signaling molecules exert distinct roles during development, influencing either the expansion of epithelial progenitors, their differentiation or both. A selection of the major signaling pathways involved in the pancreatic epithelial-mesenchymal crosstalk is reviewed in the following section.

FGF signaling pathway

In the mouse embryo from E9.5 onward, different members of the *Fgf* family are expressed in the pancreatic mesenchyme, including *Fgf1, Fgf7* and *Fgf10*, whereas the *Fgf receptor 2b* (*Fgfr2b*) and *Fgfr3* are expressed in the pancreatic epithelium (Arnaud-Dabernat et al., 2007; Bhushan et al., 2001; Miralles et al., 1999; Ye et al., 2005). FGF10 is among the best characterized signaling molecules in the pancreatic mesenchyme and its function has been extensively studied both *ex vivo* in pancreatic explants as well as *in vivo* in the mouse. *Fgf10-/-* embryos display severe pancreatic hypoplasia both dorsally and ventrally, due to failure of proliferation of specified Pdx1+ progenitors (Bhushan et al., 2001). Consistently, the pancreas in *Fgfr2b-/-* embryos is smaller than in wild-type littermates and pancreatic ductal branching as well as duct cell proliferation are significantly reduced, even though to a lesser extent than in the absence of FGF10

(Pulkkinen et al., 2003). Further investigation showed that *Fgf10* is required for the maintenance of *Pdx1* expression and induction of *Ptf1a* in MPCs (Jacquemin et al., 2006). A more recent study identified a relationship between FGF10 and another the pancreatic TF, Sox9, which is required for progenitor proliferation and survival (Seymour et al., 2012). Specifically, mesenchymal-derived FGF10, transduced through the FGFR2B receptor, sustains *Sox9* expression in the epithelium, which in turn is required for *Fgfr2b* expression and, therefore, FGF10 response (Seymour et al., 2012). Mechanistically, this process involves a SOX9/FGF10/FGFR2B feed-forward loop in the maintenance of pancreatic identity, and its perturbation results into loss of pancreatic cell identity and conversion into liver fate (Seymour et al., 2012). Interestingly, such a functional relationship between FGF10 and Sox9 is conserved in zebrafish embryos, where combined action of FGF10 and Sox9 is essential for the morphogenesis of the hepatopancreatic ducts and prevents pancreatic, hepatic and intestinal cell plasticity (Dong et al., 2007; Manfroid et al., 2007).
Unlike the other FGFR-driven signaling, FGFR3 has been reported to inhibit the expansion of the pancreatic epithelium (Arnaud-Dabernat et al., 2007). Likely, these differences in activities reflect temporal changes in expression of the signaling components and the dynamic nature of cellular competence in response to extracellular signals.

TGFβ / BMP signaling pathway

The TGFβ ligands, *Tgfβ1*, *Tgfβ2* and *Tgfβ3,* are expressed in the mouse pancreatic epithelium starting from E12.5, becoming subsequently restricted to the acinar cells (Crisera et al., 2000; Gittes, 2009). At the same embryonic stage, the TGFβ receptors, *Tgfbr1* and *Tgfbr2,* show an expression pattern similar to their ligands, but localize to ductal cells as development proceeds (Gittes, 2009; Tulachan et al., 2007). In addition, *Tgfbr1* is also expressed in the pancreatic mesenchyme at early stages (Tulachan et al., 2007). Multiple studies supported a role for TGFβ in the process of delamination of

endocrine progenitors from the trunk compartment into the surrounding mesenchyme and consequent coalescence into islets (Miralles et al., 1998a; Sanvito et al., 1995; Tulachan et al., 2007). In particular, TGFβ1 has been shown to control the activity of Matrix metalloproteinase-2 (MMP-2) and islet morphogenesis *in vitro* (Miralles et al., 1998a).

Activins and their receptors are important in the early foregut endoderm for pancreatic specification (see above), but also for endocrine differentiation at later stages (Maldonado et al., 2000; Verschueren et al., 1995). In particular, the ligands Activins A and B localize to the developing endocrine cells, being enriched in the glucagon-expressing α-cells (Maldonado et al., 2000). *Activin receptor type IIB* (*Acvr2b*) and *Activin receptor type IIA* (*Acvr2a*) mutant mice display pancreatic hypoplasia with reduced number of endocrine cells, which in turn lead to hypoplastic islets (Kim et al., 2000). Moreover, *ex vivo* treatment of mouse pancreatic explants with recombinant Activin results in reduced branching morphogenesis and an increased number of insulin-expressing cells (Demeterco et al., 2000; Ritvos et al., 1995). Consistently, when pancreatic explants are treated with Follistatin, a well-known Activin antagonist, cells undergo enhanced exocrine differentiation at the expenses of the endocrine cell lineage (Miralles et al., 1998b).

BMP molecules are also expressed in the developing pancreas and have been suggested to regulate the development of both pancreatic epithelium and mesenchyme (Ahnfelt-Rønne et al., 2010; Dichmann et al., 2003; Goulley et al., 2007). Bmp signaling is repeatedly used in this developmental process, exerting different, sometimes even opposite, actions. At early developmental stages, BMP is antagonistic to pancreatic fate and acts as a pro-hepatic factor, as seen in multiple vertebrate species, while few hours later it has opposite effects, promoting adoption of pancreatic fate in mouse foregut explants (Chung et al., 2008; Deutsch et al., 2001; Rossi et al., 2001; Spagnoli and Brivanlou, 2008; Wandzioch and Zaret, 2009). Additionally, conditional deletion of the BMP receptor 1A (ALK3) in insulin-positive cells identified a role for BMP in glucose-stimulated

insulin secretion in the mouse adult pancreas (Goulley et al., 2007). Similarly, ectopic expression of BMP inhibitors, such as *Noggin* and *Smad6*, under the *Pdx1* promoter results into glucose intolerance and diabetes, but normal pancreatic morphology (Goulley et al., 2007).

Likewise, in mouse or human ES cultures differentiating along the pancreatic cell lineage BMP exert versatile functions. While inhibition of BMP is important for inducing pancreatic fate specification from pluripotent ESCs, the late exposure of induced pancreatic progenitors to BMP inhibitors helps to ensure precise temporal activation of NEUROG3, favoring the generation of mono-hormonal insulin-secreting cells *versus* polyhormonal cells (D'Amour et al., 2006; Kroon et al., 2008; Mfopou et al., 2010; Russ et al., 2015).

The intracellular mediators of BMP and TGFβ signaling are the SMAD molecules (Massagué et al., 2005). SMAD1, SMAD5 and SMAD8 are activated by BMP and, specifically, inhibited by SMAD6, whereas SMAD2 and SMAD3 are downstream of TGFβ isoforms and Activin. Beside a short window of time at E8.5, when phosphorylated (active) (p)-Smad1,5,8 has been reported in the foregut endoderm (Wandzioch and Zaret, 2009), BMP-mediated Smad activation seems entirely restricted to the mesenchyme of both chicken and mouse pancreas at subsequent stages (Ahnfelt-Rønne et al., 2010). Consistently, *ex vivo* studies in mouse explants indicate that BMP signaling is primarily required in the mesenchyme and that the effects on the epithelium might be indirect (Ahnfelt-Rønne et al., 2010).

Together these findings underscore once more the importance of time in signaling events and changes of cellular competence in response to extracellular signals. It is also unclear how BMPs mediate such a wide variety of responses. It may be, for instance, a result of diversity in the components of signal transduction or crosstalk with other pathways. Possibly, better *in vivo* models for fine-tuned spatio-temporal control and precise

manipulation of signaling pathways at key developmental-equivalent stages are required to answer these questions.

Notch signaling pathway

Notch receptors and ligands are expressed in the developing pancreas starting from E9.5 in the mouse pancreatic epithelium, with the exception of Notch 3 and Notch 4, which are in mesenchymal and endothelial cells, respectively (Lammert et al., 2000).

At early stages, Notch signaling is necessary to maintain proliferation of the undifferentiated pancreatic progenitors and prevent endocrine and exocrine differentiation, acting in a conserved fashion across species (Ahnfelt-Rønne et al., 2007; Apelqvist et al., 1999; Jensen et al., 2000; Murtaugh et al., 2003). Null mutant mice for either Notch ligand genes, such as *Dll1*, or for Notch target genes, like *Rbpj-k* and *Hes1*, display pancreatic hypoplasia due to accelerated and increased endocrine differentiation (Apelqvist et al., 1999; Jensen et al., 2000). Moreover, constitutive expression of an active intracellular domain of *Notch1* under the control of the *Pdx1* promoter results into failure of endocrine and exocrine differentiation, which is accompanied by expansion of the pancreatic progenitor number (Murtaugh et al., 2003).

An interplay between Notch and FGF signaling has been reported, whereby FGF10 would maintain active Notch signaling to control epithelial cell proliferation and differentiation (Hart et al., 2003; Norgaard et al., 2003). Specifically, Notch signaling controls proliferation of pancreatic progenitors downstream of FGF10 through Hes1 transcriptional control of *Cdkn1c* (Georgia et al., 2006; Miralles et al., 2006; Norgaard et al., 2003). In contrast, endocrine and exocrine cells are induced within ductal epithelium *via* Notch signaling by lateral inhibition (Ahnfelt-Rønne et al., 2012; Apelqvist et al., 1999; Murtaugh et al., 2003). The mechanism through which Notch controls endocrine differentiation involves a Hes1-dependent suppression of *Ngn3* expression, whereas its modulation of acinar

differentiation depends on the inhibition of PTF1a (Ahnfelt-Rønne et al., 2012; Esni et al., 2004; Murtaugh et al., 2003).

Hedgehog signaling pathway

Hh signaling needs to be tightly-regulated throughout pancreas development. Pancreas fate specification requires suppression of Hh signaling activation in the foregut endoderm (see above) (Hebrok et al., 2000; Kim et al., 1997). Subsequently in development, disrupted Hh signaling affects the development of both endocrine and exocrine cells and impairs β-cell function and mass (Hebrok et al., 2000; Kawahira et al., 2003; Kawahira et al., 2005). Efforts have been made to differentiate between Hh signaling contribution to pancreas epithelium and mesenchyme. Manipulation of the pathway in epithelial and β-cells showed a cell-autonomous role of Hh signalling in regulating endocrine mass and β-cell function (Thomas et al., 2001; Thomas et al., 2000). More recent work from Landsman and colleagues has provided direct evidence for non-autonomous roles of the Hh pathway in pancreatic development (Hibsher et al., 2016). Specifically, by deleting the receptor *Ptch1* in the pancreatic mesenchyme, Shh signalling activity is increased, due to the abrogation of the inhibitory function of Ptch1 on Smoothened. Such deregulated Hh signaling in mesenchymal cells leads to hyperplasia of the pancreatic mesenchyme along with disrupted epithelial growth and islet cellular composition (Hibsher et al., 2016). How the Hh pathway signals in the mesenchyme is unknown. It is unclear whether the epithelial defects are consequences of the mesenchymal hyperplasia, which might hamper proper mesenchymal-epithelial interactions. Finally, Hh-dependent mesenchymal cues that regulate pancreatic growth and differentiation are yet to be identified.

Retinoids signaling pathway

Vitamin A-derived RA, retinoid binding proteins and RA receptors are expressed in both pancreatic epithelium and mesenchyme during pancreas development (Arregi et al., 2016; Martín et al., 2005; Molotkov et al., 2005; Stafford and Prince, 2002; Tulachan et al., 2003). Specifically, the enzymes RDH10 and RALDH2, responsible for the production of RA, are present in the lateral plate mesoderm at E8.25, before *Pdx1* induction, and later in the pancreatic mesenchyme (Arregi et al., 2016; Martín et al., 2005; Molotkov et al., 2005; Stafford and Prince, 2002; Tulachan et al., 2003).

In vertebrate species, including zebrafish, *Xenopus* and mouse, mesoderm-derived RA signaling induces pancreatic specification within the endoderm. Deletion of *Rdh10* or *Raldh2* as well as expression of dominant-negative RA receptors result into impaired pancreatic specification within the dorsal foregut endoderm (Arregi et al., 2016; Chen et al., 2004; Martín et al., 2005; Molotkov et al., 2005; Stafford and Prince, 2002; Tulachan et al., 2003). Later in the mouse embryo, RA has been shown to control endocrine and ductal differentiation and influence ductal *versus* acinar cell fate decision (Arregi et al., 2016). Specifically, endoderm specific-*Rdh10* deletion results into pancreatic hypoplasia with impaired endocrine differentiation observed at later stages (Arregi et al., 2016). Consistently, treatment of embryonic pancreatic explants with RA promotes ductal cell differentiation with premature formation of endocrine clusters at the expense of exocrine differentiation and branching morphogenesis (Shen et al., 2007; Tulachan et al., 2003). Interestingly, in *Rdh10* and *Raldh2* null mutant embryos the dorsal pancreatic mesenchyme is also disrupted, suggesting a further role of RA signaling in formation and patterning of mesenchymal cells (Arregi et al., 2016; Martín et al., 2005). Yet, specific mesenchymal contribution of the RA signaling has not been investigated and the distinction between cell-autonomous and non-cell-autonomous contributions need to be addressed.

Epidermal growth factor (EGF) signaling pathway

EGF receptors (EGFR) and EGF ligands are present in both epithelium and mesenchyme of the developing pancreas and the pathway has a role in pancreatic morphogenesis and differentiation (Gittes, 2009; Kritzik et al., 2000). *Egfr* KO mouse embryos display impaired branching morphogenesis, due to proliferation defects, and delayed endocrine differentiation with perturbed islet formation (Miettinen et al., 2000). Moreover, overexpression of a dominant-negative form of *Egfr* under the *Pdx1*-promoter results into impaired postnatal β-cell proliferation and diabetes (Miettinen et al., 2000; Miettinen et al., 2006). In addition to *Egfr*, the *Erbb4* receptor is expressed in pancreatic epithelial cells at E12.5 and becomes restricted to the ductal epithelium by E16.5 (Kritzik et al., 2000). This receptor has been suggested to play a role in δ-cell development, since treatment of pancreatic explants with an antibody neutralizing Neuregulin 4, the Erbb4 ligand, specifically blocked δ-cell expansion (Huotari et al., 2002).

Recent work by Semb and colleagues has started shedding light into how EGFR signaling coordinates epithelial morphogenesis and cell differentiation in the pancreas (Löf-Öhlin et al., 2017). They identified an interesting and conserved mechanism whereby EGF/EGFR signaling controls β-cell differentiation *via* modulation of apical polarity in endocrine progenitor cells. Morevover, they reported sequential temporal activation of the EGFR by distinct EGF ligands during pancreas development (Löf-Öhlin et al., 2017). Specifically, during the primary transition (E9.5-E12) EGF ligand counteracts polarization of pancreatic epithelial progenitors, while later Betacellulin (BTC) ligand appears to induce β-cell commitment by reducing the apical domain size during the secondary transition. Apical domain constriction results into reduced Notch signaling, increased *Ngn3* expression and β-cell differentiation (Löf-Öhlin et al., 2017).

Hepatocyte growth factor (HGF) signaling pathway

Hgf and its receptor *Met* are expressed in the pancreatic mesenchyme and epithelium, respectively (Garcia-Ocaña et al., 2000; Otonkoski et al., 1996). HGF signaling was shown to induce β-cell formation both *in vitro* and *in vivo*, including in human fetal pancreas cultures (Garcia-Ocaña et al., 2000; Otonkoski et al., 1996). Moreover, transgenic mice expressing *Hgf* under the rat *insulin* promoter display an increase in islet number with enhanced insulin content (Garcia-Ocaña et al., 2000). Consistently, deletion of *Met* in β-cells results in mice that exhibit glucose intolerance with reduced insulin secretion and islet size (Dai et al., 2005; Roccisana et al., 2005).

Interestingly, recent findings in zebrafish suggest a role for HGF-Met signaling in pancreas morphogenesis through control of directional migration of pancreatic progenitors (Anderson et al., 2013). If a similar mechanism contributes to pancreatic branching morphogenesis in mammals remains to be tested.

Wnt signaling pathway

In the developing pancreas, Wnt signaling plays multiple roles that are spatially and temporally restricted. An early role for Wnt signaling in pancreatic fate specification has been described in *Xenopus* and mouse embryos (see above) (McLin et al., 2007; Rodríguez-Seguel et al., 2013). In the frog, canonical Wnt signaling needs to be repressed in the anterior endoderm in order to maintain foregut identity and allow pancreas and liver development (McLin et al., 2007). Once the foregut territory has been established, the non-canonical Wnt signaling regulates the pancreas *versus* liver cell fate decision in both *Xenopus* and mouse (Rodríguez-Seguel et al., 2013). In particular, non-canonical Wnt family members, such as *Wnt5a*, display a differential expression pattern between pancreatic and hepatic progenitors within the foregut endoderm (Rodríguez-Seguel et al., 2013). Consistently, Wnt5A treatment of *Xenopus* endodermal cells and mouse ESC (mESC) cells induce *Pdx1* expression (Rodríguez-Seguel et al., 2013).

Subsequently, several Wnt ligands localize in the mouse pancreatic mesenchyme, whereas Frizzled receptors (Fzd) and secreted Frizzled-Related Proteins (SFRP) are present in both the epithelium and mesenchyme (Heller et al., 2002). Mice overexpressing *Wnt1* and *Wnt5a* under the control of the *Pdx1* promoter display pancreatic agenesis and pancreatic hypoplasia, respectively, which is in line with its early role in foregut specification (Heller et al., 2002). Similarly, constitutive activation of β-catenin at early stages of pancreatic development leads to dramatic pancreatic hypoplasia (Heiser et al., 2006). By contrast, ablation of *β-catenin* in *Pdx1*-expressing pancreatic progenitors reduces epithelial proliferation and, specifically, prevents exocrine differentiation (Murtaugh et al., 2005). Consistently, inhibition of Wnt activity by expressing a soluble form of *Fzd8* under the *Pdx1* promoter results into a reduced number of proliferating pancreatic progenitor cells and organ hypoplasia, affecting both exocrine and endocrine compartments in mice (Papadopoulou and Edlund, 2005). Interestingly, Wnt signaling is also an essential mediator of pancreatic mesenchymal cell survival. Specific inactivation of canonical Wnt signalling in the pancreatic mesenchyme, using *β-catenin*$^{fl/fl}$;*Nkx3.2-Cre* Tg embryos, results in the loss of mesenchymal cells, which in turn affects the epithelium (Landsman et al., 2011). Consistently, a more recent study has identified Wnt7b ligand to be expressed in the epithelium and act as trophic factor for the mesenchyme *via* a paracrine mechanism from epithelium-to-mesenchyme (Afelik et al., 2015). Overall, because of the myriad of Wnt and Frizzled possible combinations as well as their diverse context-dependent functions, the complexity of the pathway deserves further attention.

Taken together, most of the studies discussed in this section highlight the relevance of signaling pathways, many of which act concurrently, in epithelial-mesenchymal crosstalk(s) during pancreas organogenesis. The mechanisms by which these

mesenchymal signals are coordinated and integrated to stimulate epithelial progenitor proliferation and differentiation to produce functional pancreatic cells remain elusive. Also, whether is the developing pancreatic mesenchyme a homogenous tissue or composed of different mesenchymal cell populations, resembling distinct “cellular niches”, like in the haematopoietic and nervous system, lungs and skin, is unclear (Crane et al., 2017; Kfoury and Scadden, 2015; Lattanzi et al., 2015; Roberts et al., 2017; Zepp et al., 2017). If different niches exist, what TFs or intrinsic regulators confer spatio-temporal control of gene expression within the mesenchyme during pancreatic development is unknown.

To date, only a small number of TFs have been reported in the embryonic pancreatic mesenchyme, including *Isl1*, *Nkx3.2* and *Hoxa6*, *Hoxb6* and *Hoxc6* (Ahlgren et al., 1997; Asayesh et al., 2006; Hecksher-Sørensen et al., 2004; Landsman et al., 2011; Larsen et al., 2015). *Isl1* is expressed in both pancreatic epithelium and mesenchyme and is required for dorsal mesenchyme formation (Ahlgren et al., 1997). *Nkx3.2* (a.k.a. *Bapx1*) expression is in the splenopancreatic mesenchyme, but not in the epithelium (Asayesh et al., 2006; Hecksher-Sørensen et al., 2004). Null mutant embryos for *Nkx3.2* displayed asplenia and metaplastic transformation of embryonic pancreas into intestinal-like tissue due to the loss of splenic mesenchyme condensation and separation (Asayesh et al., 2006; Hecksher-Sørensen et al., 2004). More recently, the expression of *Hox6* genes has been identified and studied in the mesenchymal compartment of the developing pancreas. Full KO embryos for all the three *Hox6* paralogs results into impaired endocrine differentiation with disruption of branching morphogenesis (Larsen et al., 2015). To fully elucidate the identity and role(s) of mesenchymal genetic regulators driving pancreas development, future investigation are required using tools which enable fine-tuned gene manipulation in the mesenchyme.

Extracellular matrix molecules

The basement membrane (BM) separates the epithelium from the surrounding mesenchyme and is crucial for epithelial tissue organization and function, including in the pancreas (Hisaoka et al., 1993). The BM is a fundamental site of interaction between epithelial cells and the ECM: the ECM mainly consists of fibronectin (FN), laminins and collagen-IV components, while the epithelium expresses multiple integrins molecules, which act as molecular hubs connecting the outside environment (mesenchyme) with the inside (epithelium) (Glentis et al., 2014; Guo and Giancotti, 2004; Hisaoka et al., 1993).

During pancreas organogenesis, the BM controls branching morphogenesis and cell differentiation (Crisera et al., 2000; Jiang et al., 1999; Jiang et al., 2001; Shih et al., 2016). Disruption of cell-ECM interactions in pancreatic explant cultures using the tetrapeptide arginyl-glycyl-aspartyl-serine (RGDS), which competitively inhibits ECM-cell interactions, blocks branch formation (Shih et al., 2016). Moreover, substrates containing Laminin 1 (*a.k.a.* Laminin 111) have been shown to induce pancreatic cell differentiation in *ex vivo* cultures (Jiang et al., 1999; Jiang et al., 2001). Consistent with this, several studies have suggested the requirement of integrins for initiation of branching morphogenesis, delamination of endocrine progenitors and islet formation. For instance, pancreatic-specific deletion of the β_1-integrin subunit leads to impaired branching morphogenesis, due to BM defects and aberrant organization of the actin cytoskeleton (Shih et al., 2016). Furthermore, $\alpha_6\beta_4$ and $\alpha_5\beta_1$ integrin, together with Netrin, a Laminin soluble form, are important for pancreatic epithelial cell adhesion and migration *in vitro* (Yebra et al., 2003). Other integrins, such as $\alpha_V\beta_5$ and $\alpha_V\beta_3$, have been shown to interact with Fibronectin, Collagen-IV and Vitronectin to maintain ECM anchorage and allow cell migration in the human fetal pancreas (Cirulli et al., 2000).

Taken together, these studies highlight the relevance of ECM and cell adhesion molecules as interfaces of signaling transmission within different compartments of the pancreas during the entire duration of its development. Microenvironmental signals transmitted via

the ECM might establish “niches” and mediate localized cellular behaviours in the embryonic pancreas.

Blood vessels

The physical interaction between the pancreatic epithelium and blood vessels is highly dynamic and changes throughout development (Azizoglu and Cleaver, 2016). During the primary transition (E9.5-E12), the pancreatic epithelium is avascular, with blood vessels only surrounding but not penetrating the epithelial buds (Pierreux et al., 2010). Starting with the secondary transition, blood vessels intercalate between the forming epithelial branches, either actively through angiogenesis or, passively, as the epithelium remodels (Pierreux et al., 2010). Moreover, the vessels follow a stereotypical pattern in their organization along the branches, becoming preferentially located near the central (trunk) epithelial cells and at a distance from the branch tips where acinar cells differentiate. This correlates with a predominant expression of the angiogenic factor Vascular Endothelial Growth Factor-A (VEGF-A) in trunk cells (Pierreux et al., 2010). In the mature organ, the branches of the pancreatic epithelium and the vascular systems closely intercalate, with densely vascularized islets connected to large vessels that run across the pancreas (Azizoglu and Cleaver, 2016). This constantly evolving relationship between blood vessels and pancreatic epithelium seems to influence different aspects of pancreas development (Azizoglu and Cleaver, 2016). While the early interaction with the aorta is crucial for pancreas fate specification (see above), at later stages endothelial cells restrict the growth of embryonic pancreas as wells as inhibit branching and differentiation (Jacquemin et al., 2006; Lammert et al., 2001; Magenheim et al., 2011a; Pierreux et al., 2010; Sand et al., 2011; Yoshitomi and Zaret, 2004). For instance, *in vitro* treatment of wild-type mouse pancreatic explants with VEGFA or VEGFR2-inhibitor leads to decreased exocrine proliferation or increased exocrine differentiation, respectively (Magenheim et al., 2011a;

Pierreux et al., 2010). Moreover, pancreatic explant cultures isolated from mouse transgenic embryos overexpressing *Vegfa* display hypervascularization with reduced exocrine differentiation (Pierreux et al., 2010). Similar effects have been reported *in vivo* in pancreata from E12.5 embryos overexpressing *Vegfa* (Magenheim et al., 2011a). In summary, blood vessels- and blood-derived signals play dynamic roles during pancreatic development either by direct signaling to the endoderm or indirectly through the mesenchyme.

Neural crest derived-cells

The neural crest (NC) cells are multipotent embryonic cells that arise from the dorsal neural tube and migrate to generate diverse ectodermal cell lineages (Young and Newgreen, 2001). Concomitant with the evagination of the pancreatic buds at E9, migrating NC cells reach the rostral foregut and, in a rostral-to-caudal migratory wave, populate the entire gut with progenitors of glial and neural cells (Young and Newgreen, 2001). Approximately at E10, NC cells enter the pancreatic mesenchyme and intermingle with the pancreatic epithelium as it branches into the mesenchyme (Plank et al., 2011). Subsequently, these NC cells differentiate into glial Schwann cells that surround mature pancreatic islets and into neurons of the autonomic nervous system, both sympathetic and parasympathetic, that regulate islet function, such as insulin and glucagon secretion (Plank et al., 2011). *Glial Cell Derived Neurotrophic Factor (GDNF),* released from the pancreatic epithelium, has been proposed to act as a neurotrophic factor guiding the migration of neural progenitors into the pancreas (Muñoz-Bravo et al., 2013).

To date, the role of NC-derived cells during pancreas development is not fully understood. Mouse mutants for two NC-specific TFs *Forkhead Box D3 (Foxd3)* and *Paired Like Homeobox 2b (Phox2b)* display loss of NC cells, which is accompanied by increased β-cell proliferation (Nekrep et al., 2008; Plank et al., 2011). Although the number is increased,

mutant β-cells display immature features, such as decreased expression of *Mafa*, *Pdx1* and *Glut2* (Plank et al., 2011). Additionally, *Phox2b(-/-)* mutant embryos display upregulation of *Nkx2.2* expression in the epithelium, suggesting a non-cell-autonomous feedback loop that links the neural crest with the pancreatic epithelium (Nekrep et al., 2008). Indeed, analysis of *Nkx2.2* KO embryos showed that signals from the pancreatic epithelium silence the expression of *Phox2b* in the NC-derived cells as they migrate into the pancreas (Nekrep et al., 2008). Perhaps, such inhibition is required for NC cells differentiation into specific cell types, like in the central nervous system. Altogether, these studies suggest that NC-derivatives negatively regulate β-cell proliferation and promote β-cell maturation.

Implications for directed differentiation of pluripotent stem cells into pancreatic β-cells

Diabetes is a degenerative disease that affects over 400 million persons worldwide (http://www.who.int/diabetes/) (Ellis et al., 2017; Sneddon et al., 2018). In type I diabetic (T1D) patients, the autoimmune destruction of pancreatic beta-cells leads to insufficient production of insulin and hyperglycemia ensues (Pociot and Lernmark, 2016; Weir and Bonner-Weir, 2013). T2D is the most common form of the disease; it develops during adulthood and is characterized by β-cell dysfunction, insulin resistance and metabolic stress, which eventually lead to β-cell mass reduction (Nolan et al., 2011; Weir and Bonner-Weir, 2013). Despite the availability of insulin as treatment to temporarily restore the glucostasis in diabetic patients, this remedy is unable to avoid both the acute dangers of hypoglycemia or the long-term complications of hyperglycemia. Eventually, the challenge of treating diabetes centers on the replacement of the destroyed β-cells. Indeed, islet and whole pancreas transplantation have become gold-standard procedures in achieving glucose control in diabetic patients (Shapiro et al., 2000; Shapiro et al., 2017).

However, the extremely limited number of suitable donor tissues severely hampers the broad application of these therapies, underscoring the need for an unlimited source of insulin-producing cells that could be used in cell-based replacement therapy.

Over the last decade, remarkable progress has been made with regard to the generation of functional β-cell equivalents from pluripotent stem cells (hES and iPS cells) and other cellular sources (Ellis et al., 2017; Sneddon et al., 2018). It has also become clear that the key to success is to closely recapitulate the path that pluripotent stem cells undertake during embryogenesis to become first definitive endoderm, then pancreatic progenitors, and finally pancreatic islet cells (Ellis et al., 2017; Sneddon et al., 2018).

In a first landmark study by D'amour *et al.* (D'Amour et al., 2005), hESC cultures were differentiated into endoderm upon combined exposure to Wnt and Activin/TGFβ signalings and, subsequently, into endocrine progenitor cells following the activation of FGF10, RA and BMP inhibition, similar to what happens in the mouse embryo. This study has not only paved the way for making β-like cells in the lab, but also revealed that pancreatic signals, which create the micro-environment controlling β-cell development, are conserved between mouse and human (D'Amour et al., 2005). Subsequent studies have improved each step of this differentiation protocol, including the initial fate specification, maturation and proliferation (D'Amour et al., 2006; Kroon et al., 2008; Nostro et al., 2011; Pagliuca et al., 2014; Rezania et al., 2013; Russ et al., 2015). For example, compound screens of molecules have been performed to direct ES cells to the endodermal lineage and to induce pancreatic progenitor specification (Borowiak et al., 2009; Chen et al., 2009). Also, co-culturing human and murine ESC-derived pancreatic and endocrine progenitors with primary pancreatic mesenchymal cell has been shown to promote progenitor expansion without altering differentiation potential (Sneddon et al., 2012). The same study subsequently tested the ability of sixteen growth factors to mimic the co-culture effect but none of them, alone or in combination, reproduced the co-culture effect at the same

magnitude, suggesting the involvement of cell-cell contact in this effect (Sneddon et al., 2012).

To date, the *ex vivo* generation of β-cell equivalents that possess physiological features of their native counterparts remains a challenge: hESC-derived insulin-producing cells often display features of immature β-cells and are frequently polyhormonal (Sneddon et al., 2018). Nevertheless, once grafted *in vivo* in the mouse, these immature β-like cells are able to further differentiate and protect animals from chemical-induced diabetes (Kroon et al., 2008; Sneddon et al., 2018). More recent efforts have been placed on generating *ex vivo* single hormone-positive β-cells capable of glucose-stimulated insulin secretion and perfectly replicating all aspects of endogenous β-cells (Pagliuca et al., 2014; Rezania et al., 2013; Russ et al., 2015).

Beside further refinement of the cytokine cocktail, recent studies have all underscored the benefits of three-dimensional cell culture system, which allows the generation of endocrine clusters that are perhaps closer to the endogenous islet (Pagliuca et al., 2014; Rezania et al., 2013; Russ et al., 2015). Indeed, a key determinant of β-cell function is cell-cell communication, among endocrine cells as well as with the cells in the islet niche, which is composed of multiple non-endocrine cell types, including endothelial, pericytes, neuronal, and mesenchymal cells (Aamodt and Powers, 2017; Brissova et al., 2014; Coronel and Stabler, 2013; Sasson et al., 2016). Given the specialized relationship between β-cells and supporting cells in their natural environment, future efforts are required to define elements of the endogenous β-cell *niche* for improving survival and function of hESC-derived β-cells *in vitro*.

Open questions and future directions

The interplay between pancreatic epithelium and all cellular components of the pancreatic microenvironment has been shown to be critical for the formation of a proper functional

pancreas. The understanding of the effectors and mechanisms underlying the crosstalk has tremendously helped in developing the current pancreatic differentiation protocols of pluripotent stem cells into β-cell equivalents for cell transplantation. Additionally, a comprehensive knowledge of the pancreatic microenvironment composition and its various signaling molecules will be relevant for further elucidating the pathogenesis underlying pancreatic diseases, such as cancer. PDAC is histologically characterized by an abundant stroma surrounding malignant epithelial cells that consists of a dynamic assortment of ECM components and fibroblasts, endothelial cells, immune cells, and neurons (Feig et al., 2012; Sinha and Leach, 2016). Accumulating evidence indicates that the epithelial and stromal compartments interact to enhance the aggressive nature of this disease (Feig et al., 2012; Sinha and Leach, 2016). Aberrant activation of embryonic signaling pathways is frequent in PDAC, making developmental regulators therapeutically attractive (Roy et al., 2016). Specifically, a number of pathways, including Hedgehog, Notch and TGF-beta, which are at play in the pancreatic mesenchyme during development, are also involved in mediating cross-talk between the malignant pancreatic epithelium and its associated stroma, making them unique pathways to target for the treatment of pancreatic cancer (Bailey and Leach, 2012). How similar is the composition of the pancreatic microenvironment in the embryo and in the tumor is an open question that needs to be addressed. Only very recently, transcriptome analyses of human embryonic pancreatic mesenchyme have become available (Ramond et al., 2017), making such comparison possible.

Many other questions deserve further investigation with regard to the pancreatic microenvironment in development and diseases. For instance, few examples of how mechanistically signaling pathways from the environment converge into the transcriptional cascade governing pancreatic development have been reported so far. Distinct types of interactions are likely to occur between different pancreatic cell types and surrounding

cells or ECM components, and these sets of interactions are dynamic and change with time. To date, the information on the identity and spatial distribution of these different types of interaction is elusive. Also, the degree of heterogeneity within the pancreatic mesenchyme is a critical open question that starts only now to be unravelled (Byrnes et al., 2018). Single-cell transcriptome analysis suggests indeed the existence of different mesenchymal lineages creating distinct “cellular niches” (Byrnes et al., 2018). This heterogeneity and potential region-specific cross-talks are awaiting to be further characterized by lineage tracing and functional studies. Finally, such information will enable the discovery of new paradigms on how fine-tuning the activity of different signaling pathways to achieve better differentiation and greater number of β-cell equivalents.

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FIGURE LEGEND

Figure 1. The pancreatic microenvironment during embryonic development. Schematic representation of the developing pancreatic epithelium and surrounding microenvironment. This is composed by multiple cell populations, such as fibroblast-like mesenchymal, endothelial, immune and neural crest-derived cells, and ECM components. In blue, tip pancreatic cells; in grey duct cells; in purple, delaminating endocrine progenitors.

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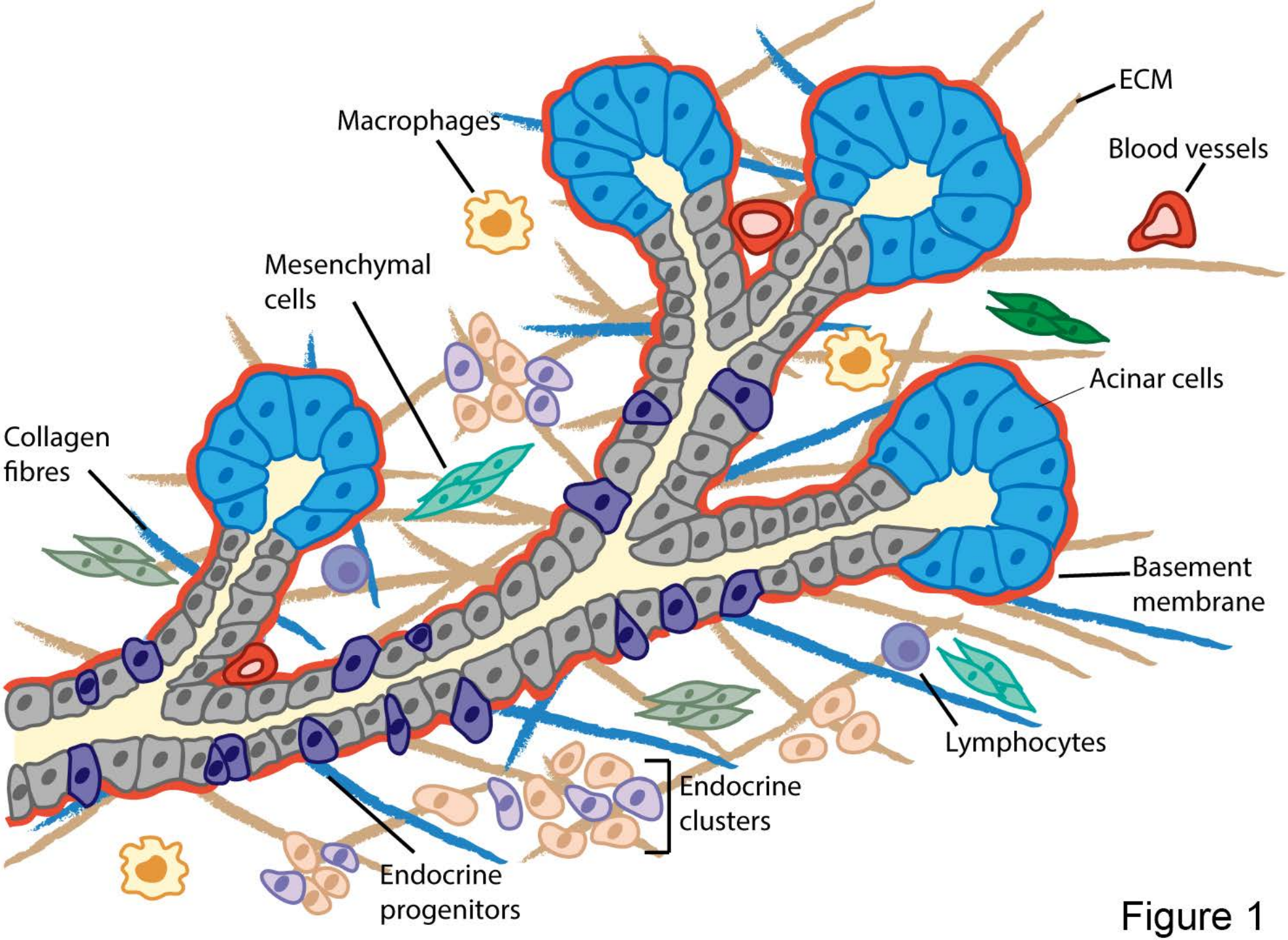
ECM
Macrophages
Blood vessels
Mesenchymal cells
Acinar cells
Collagen fibres
Basement membrane
Lymphocytes
Endocrine clusters
Endocrine progenitors

Figure 1